§ 103

1. A method for inducing stem cells to differentiate into neuronal cells comprising:

a) culturing said stem cells with basic fibroblast growth factor;

b) culturing the cells of step a) with fibroblast growth factor 8 and Sonic Hedgehog;

c) culturing the cells of step b) with brain-derived neurotrophic factor;

d) co-culturing the cells of step c) with astrocytes.

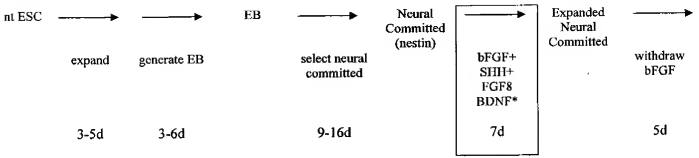
4. The method of claim 1, wherein said cells are cultured according to steps (a) through (d) for at least seven days at each step.

2003/0036195 (Studer et al.)

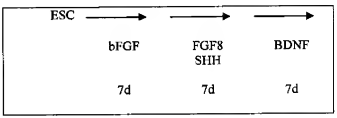
TYPE OF CELL	TEXT
GABA-ERGIC "mitogen" (Not SHH) (Not FGF8)	[0019]
SEROTONERGIC bFGF SHH FGF8	[0016]
ASTROCYTES "mitogen" SHH FGF8	[0017]
OLIGODENDROCYTES "mitogen" SHH FGF8	[0018]
DOPAMINERGIC bfgf SHH FGF8 BDNF (21 suggested)	[0015]

2003/0036195 (Studer et al.)

DOPAMINERGIC:



CLAIM 4



* optional

2003/0036195 (Studer et al.)

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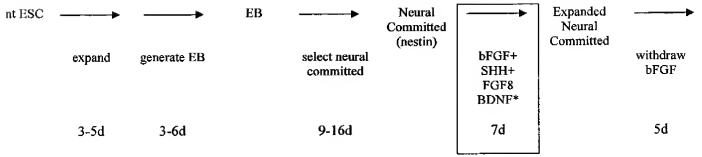
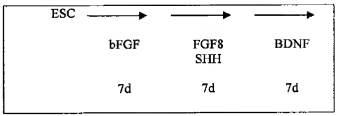


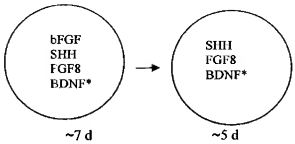
CLAIM 4



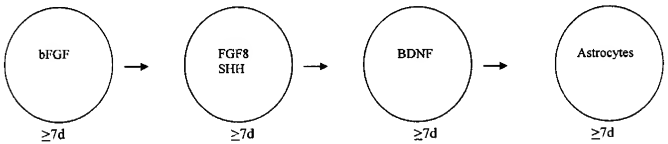
* optional

6.

ART



CLAIM 4



* optional

Stated Rationale to Reject Claim 4

"In addition, although WO 02/086073 does not explicitly teach at least 7 days for each step as recited in instant claim 4, each step and each stage of the culture conditions of WO 02/086073 require 6-9 days and the whole culture procedures take more than one month (see p. 27-29, in particular), **which is within the limitations of the instant claim 4**." Office Action dated March 17, 2008, page 8.

Note: The relevant part of the procedure in WO 02/086703 (in which cells are exposed to the FGF8, bFGF, SHH, and BDNF) takes only seven days. None of the other steps involved exposure to the four factors. Therefore, the art is not "within the limitations of claim 4."

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"New Matter"

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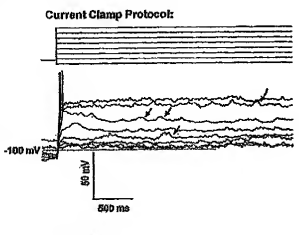
Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE,*

[Continued on next page]

(54) Title: NEURONAL DIFFERENTIATION OF STEM CELLS



(57) Abstract: The present invention relates to compositions and methods for culturing stem cells, such that neuronal differentiation can be achieved.

WO 2005/003320 A2

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the terms below are defined by the following meanings:

"Stem cell" refers to a cell that can give rise to at least two cell types of the ectodermal lineage. A "MAPC" is one type of stem cell. Another is an "embryonic stem cell."

"MAPC" is an acronym for a multipotent adult progenitor cell. It refers to a non-embryonic stem cell that can give rise to cell lineages of all three germ layers upon differentiation. See PCT/US00/21387, published as WO 01/11011, and filed as U.S. Application Serial No. 10/048,757 (specifically incorporated by reference for the description of MAPC isolation, characterization and preparation) and PCT/US02/04652, published as WO 02/064748, and filed as U.S. Application Serial No. 10/467,963 (specifically incorporated by reference for the description of MAPC isolation, characterization and preparation).

"Germ layers" are the three primary layers formed as a result of gastrulation in early stage embryos, consisting of endoderm, mesoderm and ectoderm. Embryonic germ layers are the source from which all tissues and organs derive. The endoderm is the source of, for example, pharynx, esophagus, stomach, intestine and associated glands (e.g., salivary glands), liver, epithelial linings of respiratory passages and gastrointestinal tract, pancreas and lungs. The mesoderm is the source of, for example, smooth and striated muscle, connective tissue, vessels, the cardiovascular system, blood cells, bone marrow, skeleton, reproductive organs and excretory organs. Ectoderm is the source of, for example, epidermis (epidermal layer of the skin), sensory organs, the entire nervous system, including brain, spinal cord, and all the outlying components of the nervous system.

"Multipotent" refers to the ability to give rise to more than one differentiated cell type. MAPCs have extensive multipotency, in that they can give rise to cell lineages of all three germ layers (i.e., endoderm, mesoderm and ectoderm) upon differentiation.

"Progenitor cells" are cells produced during differentiation of a stem cell and have some, but not all, of the characteristics of their terminally differentiated progeny. Defined progenitor cells are committed to a lineage, but not to a specific or terminally differentiated cell type. The term "progenitor" as used in the acronym "MAPC" does not limit these cells to a particular lineage.

In general, stem cells of the present invention comprise those having the capacity for neuronal differentiation.

In one embodiment, the stem cells are MAPCs (Jiang, Y. et al, 2002). MAPCs derived from human, mouse, rat or other mammals appear to be the only normal, non-malignant, somatic cell (i.e., non-germ cell) known to date to express very high levels of telomerase even in late passage cells. The telomeres are extended in MAPCs and they are karyotypically normal. Because MAPCs injected into a mammal can migrate to and assimilate within multiple organs, MAPCs are self-renewing stem cells. As such, they have utility in the repopulation of organs, either in a self-renewing state or in a differentiated state compatible with the organ of interest. They have the capacity to replace cell types that could have been damaged, died, or otherwise might have an abnormal function because of genetic or acquired disease.

Human MAPCs are described in U.S. Application Serial No. 10/048,757 (see page 8, lines 23-32; p.9, lines 1-22; p.21, lines 19-32; p.22, lines 1-27; p.25, lines 20-31; pages 26 through p.28, lines 1-13, 20-25; p.29, lines 1-21) and U.S. Application Serial No. 10/467,963 (see p.9, lines 29-32; p.10, lines 1-25), specifically incorporated by reference for the characterization of MAPCs.

Methods of MAPC isolation are described in U.S. Application Serial No. 10/048,757 (p.10, lines 17-32; p.11, lines 1-12; p.22, lines 29-32; p.23, lines 1-32; p.24, lines 1-28; p.71, lines 28-32; p.72 through p.74, lines 1-27) and U.S. Application Serial No. 10/467,963 (p.26, lines 13-34; p.27 through p.28, lines 1-27), specifically incorporated by reference for the methods of isolation described. Methods of MAPC culture are also described in U.S. Application Serial No. 10/048,757 (p.23, lines 25-32) and U.S. Application Serial No. 10/467,963 (p.26, lines 18-29), specifically incorporated by reference for the culture methods described.

Stem cells used in the present invention can also include embryonic stem cells (Lebkowski, J.S. et al, 2001). The quintessential stem cell is the embryonic stem (ES) cell, as it has unlimited self-renewal and pluripotent differentiation potential (Thomson, J. *et al.* 1995; Thomson, J.A. *et al.* 1998; Shamblott, M. *et al.* 1998; Williams, R.L. *et al.* 1988; Orkin, S. 1998; Reubinoff, B.E., *et al.* 2000). These cells are derived from the inner cell mass (ICM) of the pre-implantation

(10/648,757)

cells for therapies directed to the majority of the population (Wadman, M., Nature (1999) 398: 551).

Using cells from the developed individual, rather than an embryo, as a source of autologous or allogeneic stem cells would overcome the problem of tissue incompatibility associated with the use of transplanted embryonic stem cells, as well as solve the ethical dilemma associated with embryonic stem cell research. The greatest disadvantage associated with the use of autologous stem cells for tissue transplant thus far lies in their limited differentiation potential. A number of stem cells have been isolated from fully-developed organisms, particularly humans, but these cells, although reported to be multipotent, have demonstrated limited potential to differentiate to multiple cell types.

Thus, even though stem cells with multiple differentiation potential have been isolated previously by others and by the present inventors, a progenitor cell with the potential to differentiate into a wide variety of cell types of different lineages, including fibroblasts, osteoblasts, chondrocytes, adipocytes, skeletal muscle, endothelium, stroma, smooth muscle, cardiac muscle and hemopoietic cells, has not been described. If cell and tissue transplant and gene therapy are to provide the therapeutic advances expected, a stem cell or progenitor cell with the greatest or most extensive differentiation potential is needed. What is needed is the adult equivalent of an embryonic stem cell.

Summary of the Invention

The present invention provides an isolated multipotent mammalian stem cell that is surface antigen negative for CD44, CD45, and HLA Class I and II. The cell may also be surface antigen negative for CD34, Muc18, Stro-1, HLA-class-I and may be positive for oct3/4 mRNA, and may be positive for hTRT mRNA. In particular, the cell may be surface antigen negative for CD31, CD34, CD36, CD38, CD45, CD50, CD62E and CD62P, HLA-DR, Muc18, STRO-1, cKit, Tie/Tek, CD44, HLA-class I and 2-microglobulin and is positive for CD10, CD13, CD49b, CD49e, CDw90, Flk1, EGF-R, TGF-R1 and TGF-R2, BMP-R1A, PDGF-R1a and PDGF-R1b. The present invention provides an isolated multipotent non-embryonic, non-germ cell line cell that expresses

transcription factors oct3/4, REX-1 and ROX-1. It also provides an isolated multipotent cell derived from a post-natal mammal that responds to growth factor LIF and has receptors for LIF.

The cells of the present invention described above may have the capacity to be induced to differentiate to form at least one differentiated cell type of mesodermal, ectodermal and endodermal origin. For example, the cells may have the capacity to be induced to differentiate to form cells of at least osteoblast, chondrocyte, adipocyte, fibroblast, marrow stroma, skeletal muscle, smooth muscle, cardiac muscle, endothelial, epithelial, hematopoietic, glial, neuronal or oligodendrocyte cell type. The cell may be a human cell or a mouse cell. The cell may be from a fetus, newborn, child, or adult. The cell may be derived from an organ, such as from marrow, liver or brain.

The present invention further provides differentiated cells obtained from the multipotent adult stem cell described above, wherein the progeny cell may be a bone, cartilage, adipocyte, fibroblast, marrow stroma, skeletal muscle, smooth muscle, cardiac muscle, endothelial, epithelial, endocrine, exocrine, hematopoietic, glial, neuronal or oligodendrocyte cell. The differentiated progeny cell may be a skin epithelial cell, liver epithelial cell, pancreas epithelial cell, pancreas endocrine cell or islet cell, pancreas exocrine cell, gut epithelium cell, kidney epithelium cell, or an epidermal associated structure (such as a hair follicle). The differentiated progeny cell may form soft tissues surrounding teeth or may form teeth.

The present invention provides an isolated transgenic multipotent mammalian stem cell as described above, wherein genome of the cell has been altered by insertion of preselected isolated DNA, by substitution of a segment of the cellular genome with preselected isolated DNA, or by deletion of or inactivation of at least a portion of the cellular genome. This alteration may be by viral transduction, such as by insertion of DNA by viral vector integration, or by using a DNA virus, RNA virus or retroviral vector. Alternatively, a portion of the cellular genome of the isolated transgenic cell may be inactivated using an antisense nucleic acid molecule whose sequence is complementary to the sequence of the portion of the cellular genome to be inactivated. Further, a

Allowed Claims

11/238,234 (CONT 10/048,757)

99. A cell culture comprising isolated expanded human multipotent, non-embryonic, non-germ, cells that can differentiate into at least one cell type of each of the endodermal, ectodermal, and mesodermal embryonic lineages and express telomerase, said cells having undergone at least 10-40 cell doublings in culture.

100. A cell culture comprising isolated expanded human multipotent, non-embryonic, non-germ, cells that can differentiate into at least one cell type of each of the endodermal, ectodermal, and mesodermal embryonic lineages, express telomerase, and have been obtained by culture of non-embryonic, non-germ tissue, the telomerase-expressing cells having undergone at least 10-40 cell doublings in culture.

101. The cell culture of claim 99 or 100 wherein the telomerase-expressing cells have undergone 10 cell doublings.

102. The cell culture of claim 99 or 100 wherein the telomerase-expressing cells have undergone 20 cell doublings.

103. The cell culture of claim 99 or 100 wherein the telomerase-expressing cells have undergone 30 cell doublings.

104. The cell culture of claim 99 or 100 wherein the telomerase-expressing cells have undergone 40 cell doublings.

105. The cell culture of claim 99 or 100, wherein the cells are genetically modified.

106. The cell culture of claim 105, wherein the modification comprises introducing a selectable or screenable marker gene into the cells.

107. A pharmaceutical composition comprising the telomerase-expressing cells of claim 99 or 100, in a pharmaceutically acceptable carrier, the telomerase-expressing cells obtained from the cell culture of claim 99 or 100 .